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A novel fusion toxin derived from an EpCAM-specific designed ankyrin repeat protein has potent antitumor activity

Martin-Killias, P ; Stefan, N ; Rothschild, S ; Plückthun, A ; Zangemeister-Wittke, U

Abstract: PURPOSE: Designed ankyrin repeat proteins (DARPin) hold great promise as a new class of binding molecules to overcome the limitations of antibodies for biomedical applications. Here, we assessed the potential of an epithelial cell adhesion molecule (EpCAM)-specific DARPin (Ec4) for tumor targeting as a fusion toxin with *Pseudomonas aeruginosa* exotoxin A. **EXPERIMENTAL DESIGN:** DARPin Ec4 was genetically fused to a truncated form of *Pseudomonas aeruginosa* exotoxin A (ETA) and expressed in *Escherichia coli*. The cytotoxicity of Ec4-ETA was measured against tumor cell lines of various histotypes in vitro. Tumor localization and antitumor activity were determined in mice bearing 2 different EpCAM-positive tumor xenografts. **RESULTS:** Ec4-ETA expressed very well in soluble form in the cytoplasm of *E. coli* and yielded up to 40 mg after purification per liter of culture. The protein was monomeric and the disulfides of ETA formed spontaneously. Ec4-ETA bound to EpCAM with low nanomolar affinity, similar to free Ec4. Furthermore, it was highly cytotoxic against various EpCAM-positive tumor cell lines in vitro with IC(50) values less than 0.005 pmol/L. This effect was competed by free Ec4, but not by unspecific DARPins. Upon systemic administration in athymic mice, Ec4-ETA efficiently localized to EpCAM-positive tumors to achieve maximum accumulation 48 to 72 hours after injection, whereas an irrelevant control fusion toxin did not accumulate. Tumor targeting with Ec4-ETA resulted in a strong antitumor response including complete regressions in some animals. **CONCLUSIONS:** Our data show for the first time the potential of DARPins for the generation of protein therapeutics for tumor targeting, and that Ec4-ETA deserves attention for clinical development. ©2010 AACR.

DOI: <https://doi.org/10.1158/1078-0432.CCR-10-1303>

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ZORA URL: <https://doi.org/10.5167/uzh-43147>

Journal Article

Accepted Version

Originally published at:

Martin-Killias, P; Stefan, N; Rothschild, S; Plückthun, A; Zangemeister-Wittke, U (2011). A novel fusion toxin derived from an EpCAM-specific designed ankyrin repeat protein has potent antitumor activity. *Clinical Cancer Research*, 17(1):100-110.

DOI: <https://doi.org/10.1158/1078-0432.CCR-10-1303>

Clinical Cancer Research



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Clin Cancer Res Published OnlineFirst November 12, 2010.

Updated version	Access the most recent version of this article at: doi: 10.1158/1078-0432.CCR-10-1303
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A Novel Fusion Toxin Derived from an EpCAM-specific Designed Ankyrin Repeat Protein has Potent Anti-tumor Activity

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Running title: Tumor targeting with a DARPIn-toxin fusion protein

Key words: Cancer therapy, Designed Ankyrin Repeat Proteins, EpCAM, fusion
toxin, tumor targeting

Grant support: Swiss National Science Foundation for UZW and AP (310030-
119859), Krebsliga of the Kanton Zürich for UZW and AP.

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Translational Relevance

Antibodies or antibody fragments are widely used as targeting moiety for the delivery of cytotoxic drugs to tumors. However, many of these constructs have limitations due to their poor expression and aggregation tendency. To overcome these limitations, we used for the first time Designed Ankyrin Repeat Proteins (DARPs) as non-IgG scaffolds with favorable biophysical properties and much higher expression yield in *E. coli* and systematically tested their potential for tumor-targeted delivery of a highly potent biotoxin in preclinical studies. The fusion toxin Ec4-ETA" recognizing the carcinoma-associated antigen EpCAM was potently cytotoxic against carcinoma cell lines of various histotypes *in vitro*. In athymic mice targeting of human carcinoma xenografts with Ec4-ETA" resulted in a strong anti-tumor response including complete regressions. This demonstrates that DARPs are well suited for tumor targeting and that the fusion toxin Ec4-ETA" holds promise for clinical development.

Abstract

Purpose: Designed Ankyrin Repeat Proteins (DARPin)s hold great promise as a new class of binding molecules to overcome the limitations of antibodies for biomedical applications. Here, we assessed the potential of an EpCAM-specific DARPin (Ec4) for tumor targeting as a fusion toxin with *Pseudomonas* exotoxin A.

Experimental design: DARPin Ec4 was genetically fused to a truncated form of exotoxin A (ETA") and expressed in *E. coli*. The cytotoxicity of Ec4-ETA" was measured against tumor cell lines of various histotypes *in vitro*. Tumor localization and anti-tumor activity were determined in mice bearing two different EpCAM-positive tumor xenografts.

Results: Ec4-ETA" expressed very well in soluble form in the cytoplasm of *E. coli* and yielded up to 40 mg after purification per liter culture. The protein was monomeric and the disulfides of ETA" formed spontaneously. Ec4-ETA" bound to EpCAM with low nanomolar affinity, similar to free Ec4. Furthermore, it was highly cytotoxic against various EpCAM-positive tumor cell lines *in vitro* with IC₅₀ values less than 0.005 pM. This effect was competed by free Ec4, but not by unspecific DARPins. Upon systemic administration in athymic mice, Ec4-ETA" efficiently localized to EpCAM-positive tumors to achieve maximum accumulation 48-72 h after injection, whereas an irrelevant control fusion toxin did not accumulate. Tumor targeting with Ec4-ETA" resulted in a strong anti-tumor response including complete regressions in some animals.

Conclusions: Our data demonstrate for the first time the potential of DARPins for the generation of protein therapeutics for tumor targeting, and that Ec4-ETA" deserves attention for clinical development.

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Introduction

The concept of tumor-targeted therapy is based on the use of conjugates consisting of ligands binding to tumor-associated antigens or growth factor receptors which deliver cytotoxic agents selectively to tumors, while sparing normal tissues from destruction (1). These agents include radioisotopes, small organic compounds, antisense oligonucleotides and protein toxins. All of them exert different modes of action, compared to standard chemotherapy, and thus might be particularly useful to combat drug-resistant cancer. Nonetheless, it remains to be shown in every single case whether a tumor-specific localization actually occurs and what profile of anti-tumor action compared to side effects is seen.

Immunotoxins are a class of conjugates in which antibodies or antibody fragments are chemically linked to protein toxins, whereas in the more advanced constructs targeting ligand and toxin are genetically fused (2,3). The most popular toxins used for this purpose are diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A (ETA) (4), which both act by irreversibly inhibiting protein synthesis in cells. In ETA-based fusion toxins a truncated variant lacking the cell binding domain and carrying a C-terminal KDEL peptide (denoted here ETA") is commonly used (5). Currently, several of these fusion toxins are in clinical trials for the treatment of lymphomas, leukemias (6,7), mesothelioma and cancers of the ovary, pancreas and bladder (8,9).

On solid tumors, well investigated targets for antibody-based therapies are members of the epidermal growth factor receptor family, such as EGFR itself and ErbB2, and certain tumor-associated carbohydrates (2,10). The epithelial cell adhesion molecule (EpCAM) has also emerged as a promising structure for targeted therapy of solid tumors. One reason is that its efficient internalization promotes access of surface bound effector molecules to intracellular targets (11-14). EpCAM is a homophilic cell adhesion molecule of 39 to 42 kDa, consisting of an extracellular

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domain with an epidermal growth factor-like and a human thyroglobulin-like domain, and a short cytoplasmic domain. Its processing by regulated intramembrane proteolysis releases a cytoplasmic tail which activates the *wnt* signaling pathway and induces transcription of c-myc and cyclins (15,16). How this mechanism contributes to tumor progression *in vivo* is unclear. EpCAM is expressed at low levels on basolateral cell surfaces of some normal epithelia (17). On the other hand, high levels of homogenously distributed EpCAM are detectable on cells of epithelial tumors (15,18), and its overexpression represents an independent prognostic marker for reduced survival in patients with breast and ovarian cancer (19,20). Recently, EpCAM was also identified as a marker of cancer-initiating cells in colon (21), breast (22), pancreatic (23) and hepatocellular carcinomas (24) providing the opportunity to target stem-like cells, which usually respond poorly to standard therapy. The favorable properties of EpCAM for cancer therapy are currently exploited in phase II clinical trials with a scFv-ETA" immunotoxin (9,11-13) which we developed previously.

The tumor-targeting moiety for the delivery of cytotoxic agents including protein toxins is usually derived from antibodies or antibody fragments, which, however, have practical limitations due to their poor expression yield and aggregation tendencies, at least for some constructs (25,26). Since for fusion toxins no other feature of the antibody than antigen binding is required, a solution might come from the use of alternative non-IgG binding scaffolds as targeting moieties (27). These can be engineered for improved specificity, affinity, and stability to increase the production yield. One such protein class are Designed Ankyrin Repeat Proteins (DARPs) (28,29). The ankyrin repeat motif consists of 33 amino acids forming a loop, a β -turn and two antiparallel α -helices connected by a tight turn. Their high stability and favorable biophysical properties provide proteins which tolerate engineering procedures usually not applicable to antibodies. Moreover, they contain

no cysteine which can instead be introduced for site-specific modifications. Thus, DARPins fulfill the requirement of almost ideal candidates for many biomedical applications including tumor targeting. Using combinatorial libraries of DARPins along with selection by ribosome and phage display, we recently generated several binders specific for EpCAM¹, and demonstrated their potential for efficient delivery of therapeutic siRNA into tumor cells (14).

Here we describe for the first time the use of a high-affinity DARPIn (Ec4) specific for EpCAM to generate a fusion toxin with ETA". Ec4-ETA" expressed very well in *E. coli*, was easily purified to high yields, and proved to be specifically cytotoxic against various EpCAM-positive tumor cell types *in vitro*. *In vivo*, fluorescence imaging and therapy studies in athymic mice demonstrated its ability to efficiently localize to subcutaneously growing tumors upon intravenous administration, and induce strong anti-tumor effects including complete regressions.

¹ Patricia Martin Killias, Nikolas Stefan, Sascha Wyss-Stoeckle, Annemarie Honegger, Uwe Zangemeister-Wittke and Andreas Plückthun. "DARPins recognizing the tumor-associated antigen EpCAM selected by phage and ribosome display". In preparation.

Material and Methods

Tumor cells

The squamous cell carcinoma cell line of the tongue CAL27 and the colorectal carcinoma cell line HT29 were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). The breast carcinoma cell line MCF7 and the non-Hodgkin's lymphoma cell line RL were obtained from ATCC (American Type Culture Collection). The small cell lung carcinoma cell line SW2 was maintained in our laboratory. All cells were cultured in Dulbecco's modified medium (DMEM) (Sigma, Buchs, Switzerland), supplemented with 10% fetal calf serum (Amimed, Bioconcept, Allschwil, Switzerland), 100 units/ml of penicillin and 100 µg/ml of streptomycin (Sigma). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. All cells were tested negative for mycoplasma using MycoAlert (Lonza, Basel, Switzerland).

Construction, expression and purification of DARPIn-ETA" fusion toxins

The EpCAM-specific high-affinity DARPIn Ec4 was selected from a DARPIn library as described¹, the control DARPins off7 (targeting the maltose binding protein) and E3_5 (an unselected member of the N3C library) have been described elsewhere (29,30). The sequences encoding the DARPins were inserted via *Bam*HI and *Hind*III upstream into an expression vector derived from pQE30, containing a 12 amino acid linker (GSG₄)₂ and the 40 kDa truncated form of ETA₂₅₂₋₆₀₈KDEL (ETA") which was cloned as described (11,31). ETA comprises residues Glu252-Pro608 (numbering of the mature protein), fused to a C-terminal his tag followed by KDEL (denoted ETA₂₅₂₋₆₀₈KDEL or ETA").

For purification and detection the construct in addition contains an MRGS-His₆ tag at the N terminus. The DARPIn-ETA" fusion proteins were expressed in soluble form in the *E. coli* strain BL21(DE3) (Stratagene, La Jolla, USA). Cultures

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were harvested 4 h after induction with 1 mM IPTG. For purification the bacteria were resuspended in TBS₄₀₀ (50 mM Tris, 400 mM NaCl, pH 7.4 at 4°C) with 20 mM imidazole and lysed with a TS 1.1 kW cell disruptor (Constant Systems Ltd., Northants, United Kingdom). Upon centrifugation (48000 *g*, 30 min at 4°C) and filtration (pore size 0.22 µm), the fusion toxins present in the clear supernatant were purified by immobilized metal ion affinity chromatography (IMAC) using Ni-NTA superflow (Qiagen, Hilden, Germany).

Endotoxin removal

For *in vivo* application, the DARPIn-ETA" fusion toxins were further purified to eliminate endotoxin. To this end, an additional washing step with 150 column volumes phosphate buffered saline (PBS) containing 20 mM imidazole and 0.1% Triton-X-114 was performed during Ni-NTA purification, followed by size exclusion chromatography using a Superdex 200 10/300 GL column (GE Healthcare, Zürich, Switzerland). Monomeric fractions were further depleted of residual endotoxin by passage over an EndoTrap Red column (Hyglos, Regensburg, Germany), and the final endotoxin content was determined using a Limulus amoebocyte lysate (LAL) endochrome kit (Charles River, Sulzfeld, Germany).

Measurement of EpCAM-binding affinity

Surface plasmon resonance

The EpCAM-binding affinities of Ec4 and Ec4-ETA" were measured by surface plasmon resonance using a ProteOn XPR36 (Bio-Rad Laboratories, Hercules, USA) instrument. For ProteOn measurements one ligand channel of a Neutravidin (NLC) Sensor Chip was coated with 500 resonance units (RU) of the extracellular domain of EpCAM (residues 1 to 242 of the mature protein) biotinylated using a C-terminal AviTag. Kinetic data were obtained by parallel injection of different concentrations of

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Ec4 or Ec4-ETA" ranging from 0.32 to 31.6 nM at a buffer flow of 60 μ l/min in PBS pH 7.4 containing 3 mM EDTA and 0.005% Tween-20. Data evaluation was performed using the ProteOn Manager software (Bio-Rad).

Flow cytometry.

The binding affinity of Ec4 to EpCAM expressed on cells was measured by flow cytometry, and the equilibrium dissociation constant K_D was determined as the ratio of dissociation rate over association rate. MCF-7 cells were pre-incubated for 30 min at 37°C in PBS supplemented with 1% bovine serum albumin (Sigma) and 0.2% sodium azide (Fluka, Buchs, Switzerland) to inhibit internalization. For dissociation experiments, 3×10^5 cells were saturated for 1 h at 4°C with 100 nM Ec4 labeled with Alexa Fluor 488 C₅ maleimide (Molecular Probes, Invitrogen, Basel, Switzerland) via a C-terminal cysteine (Ec4cA488). Cells were centrifuged and resuspended in 1 μ M Ec4 to prevent rebinding. The mean fluorescence intensities (MFIs) were recorded at different time points ranging from 0 to 4 h. For association experiments, 3×10^5 cells were incubated with 2.5, 7.5 or 22.5 nM Ec4cA488 and measured at time points ranging from 1 to 60 min without prior washing. To correct for the small amount of non-specific binding, probably caused by Alexa Fluor 488, the association of a non-binding DARPIn labeled with Alexa Fluor 488 was measured and subtracted. Data evaluation was performed with Prism (Graphpad).

Disulfide assays

The formation of the two disulfide bonds in Ec4-ETA" after purification of protein expressed in the cytoplasm was quantified according to (32). Briefly, 1.25 nmol of fusion protein was treated with 4,4'-dithiodipyridine (4-DPS) in the presence of 8 M urea and compared to an equally treated sample reduced with sodium borohydride,

quantified by HPLC and evaluated according to a standard curve made with known amounts of cysteine.

Cytotoxicity assays

The specific cytotoxic activities of the DARPIn-ETA" fusion toxins were assessed by measuring cell viability in standard colorimetric XTT assays (XTT = (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2*H*-tetrazolium-5-carboxanilide) sodium salt) (Roche, Basel, Switzerland). Briefly, EpCAM-positive or negative tumor cells were seeded at 5000 cells per well in a 96-well plate and incubated overnight at 37°C under standard cell culture conditions as described above. The fusion proteins were added to the cells at the indicated concentrations to a final volume of 100 µl. After 72 h, 50 µl of XTT reagent was added as specified by the manufacturer's protocol and cells were further incubated for 2 h. The absorbance at 450 nm was measured with a HTS 7000 plus microplate reader (Perkin Elmer, Wellesley, USA) and cell viability was calculated after subtraction of blanks (wells without cells) as the percentage of living cells in treated wells relative to untreated cells (cells without DARPIn-ETA" toxin).

For competition analysis of specificity, cells were first preincubated for 10 min with unconjugated DARPins at the concentrations indicated before Ec4-ETA" was added and viability was determined as described above.

Fluorescence labeling of DARPIn-ETA" fusion proteins with Cy5.5

Ec4-ETA" and off7-ETA" were incubated with a 3-fold molar excess of Cy5.5 NHS ester (GE Healthcare) pH 7.4 for 1.5 h at room temperature. At this relatively low pH the N-terminal amino group is favored over lysine residues at short incubation times and modest excess of dye. The samples were passed over a PD-10 column (GE Healthcare) to remove unreacted dye and exchange the buffer to 100 mM sodium-bicarbonate with 20 mM NaCl at pH 8. Monolabeled fusion proteins were separated

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from unlabeled and multiply labeled proteins by anion exchange chromatography on a MonoQ column (GE Healthcare) in 100 mM sodium-bicarbonate and 1 M NaCl at pH 8 with isocratic elution.

Animals and tumor xenografts

For *in vivo* experiments, 8-10 weeks old female athymic mice (NMRI nu/nu, Harlan Laboratories, B.V., The Netherlands or CD1, Charles River, Sulzfeld, Germany) were used. Mice were housed and maintained under specific pathogen-free conditions according to the guidelines of the veterinary offices of the Kanton Zürich and Bern. Tumors were raised by subcutaneous (s.c.) injection into the lateral flank of HT29 or SW2 cells (10^7 cells in 100 μ l PBS).

***In vivo* serum half-life of Ec4-ETA"**

Female nude mice were intravenously injected with a single dose of 30 μ g Ec4-ETA" in 100 μ l of PBS. Blood samples were collected at different time points (3, 5, 10, 20, 30 and 60 min) from the time of the injection. Serum was harvested from the blood samples and the concentration of Ec4-ETA" was determined by ELISA in comparison to a standard curve of the corresponding pure immunotoxin.

Briefly, microtiter plates were coated with anti-Pseudomonas Exotoxin A antibody (Sigma) diluted 1:2000 in PBS overnight at 4°C. Plates were blocked with PBS containing 1% BSA (bovine serum albumin) for 2 h at room temperature followed by washing four times in PBS containing 0.05% Tween 20. A standard curve was made with pure Ec4-ETA" while serum samples were diluted 1:100 and 1:1000 in PBS. One hundred microliters of standards or samples were applied and incubated for 1 hour at room temperature. After washing, anti RGS-His antibody conjugated with horseradish peroxidase (Qiagen) diluted 1:2000 was incubated for 1 h at room

temperature. After washing, plates were developed using 3,3'-5,5'-tetramethylbenzidine (TMB) for 15 min and the absorbance was measured at 450 nm

***In vivo* fluorescence imaging**

Ten days after s.c. tumor cell injection, mice were intravenously (i.v) injected with 30 µg of Ec4-ETA" or off7-ETA" conjugated with the fluorescent dye Cy5.5 (n=3 for each group). *In vivo* imaging was performed 6, 24, 48, 72, and 96 h after injection. During imaging mice were anaesthetized by intraperitoneal (i.p.) injection of body-weight adapted doses of 10% ketamine and 2% xylazin. In addition, 48 h after i.v. injection one mouse of each group was euthanized and fluorescent images of dissected organs were obtained. Images were acquired using the NightOwl II NC100, Type LB 893, 2006 imaging system (Berthold Technologies, Bad Wildbad, Germany) with an exposure time of 60 s. For colocalization of the fluorescent image on the animal body, gray scale and pseudocolor images were merged. Quantification of signal intensity in all animals was performed with WinLight32 Software.

Anti-tumor activity *in vivo*

Mice bearing established HT29 tumors of 50 to 100 mm³ in size were i.v. injected on days 1, 3 and 5 with either 30 µg or 20 µg Ec4-ETA" or with 30 µg off7-ETA" in 100 µl PBS. Treatment with the lower dose (20 µg) of Ec4-ETA" was repeated again on days 9, 13 and 15. Mice bearing established SW2 tumors of 40 to 80 mm³ in size were i.v. injected on days 1, 3, 5, 8, 10 and 12 with 20 µg Ec4-ETA" or 20 µg off7-ETA" in 100 µl PBS. For both tumor models mice treated with PBS at the same time points were used as control.

Animals were monitored for tumor growth by caliper measurement of the shortest diameter and the longest perpendicular diameter. Tumor volume was calculated according to the formula (short diameter)² x (long diameter) x 0.4. Mice were

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euthanized when tumors reached a volume of 1500 mm³ or when tumors showed skin ulcerations.

***In vivo* toxicity determination**

During treatment with the fusion toxins animals were controlled daily for weight loss or other signs of toxicity and discomfort (apathy, ungroomed appearance, dehydration, etc). Liver toxicity was assessed post mortem by measuring alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity in plasma samples. Blood samples from 4 mice treated with PBS and 4 mice treated with 30 µg Ec4-ETA" three times every second day were collected 24 h after the final injection. Upon plasma separation, ALT and AST activities were measured photometrically.

Statistical analysis

All data represent the mean \pm SD. Statistical analyses of *in vivo* tumor growth were performed using the Kruskal-Wallis test. $P < 0.05$ was considered statistically significant.

Results

Expression and purification of DARPin-ETA" fusion proteins

The EpCAM-specific high-affinity DARPin Ec4¹ and the control DARPins off7 (29) and E3_5 (30) were fused via a (GSG₄)₂ linker to a truncated form of *Pseudomonas* exotoxin A (residues Glu252-Pro608, numbering of the mature protein), containing a C-terminal KDEL sequence (denoted ETA₂₅₂₋₆₀₈KDEL or ETA") to increase cytotoxicity in mammalian cells (33). For purification and detection, the constructs further contained a His₆ tag at the C-terminus in front of the KDEL sequence and a RGSHis₆ tag at the N-terminus.

All DARPin-ETA" fusion proteins were expressed in soluble form in *E. coli* at 37°C. The protein yield was up to 40 mg/liter of bacterial culture. Purification was achieved using immobilized metal ion affinity chromatography (IMAC), which for *in vivo* experiments was followed by extensive Triton X-114 washing, size exclusion chromatography and an EndoTrap column to remove endotoxin. The fusion toxins showed a band at the predicted molecular weight of around 59 kDa when analyzed on SDS-PAGE (Figure 1A), and size exclusion chromatography revealed a mainly monomeric fraction (Figure 1B). Figure 1C shows a structural model of Ec4-ETA". ETA" contains two disulfide bonds, and although the fusion toxins were expressed in the bacterial cytoplasm, and no redox shuffle system was used during purification, quantitative disulfide assays (see Materials and Methods) revealed that > 90% of the protein had both disulfide bonds formed after purification, most probably by spontaneous air oxidation (data not shown). We conclude from these findings that DARPins are well suited for the easy and cost-effective production of fusion toxins for tumor targeting.

EpCAM-binding affinity of Ec4-ETA"

To investigate whether the fusion to ETA" impaired the binding activity of DARPin Ec4 to EpCAM, the association (k_a) and dissociation rate constants (k_d) of both Ec4 and Ec4-ETA" were measured by surface plasmon resonance (SPR) (Supplementary Figure 1A and 1B). The measurements revealed that Ec4 and Ec4-ETA" have comparable rates of association (1.1×10^5 and $6.2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, respectively) and dissociation (1.8×10^{-4} and $1.3 \times 10^{-4} \text{ s}^{-1}$, respectively), resulting in similar equilibrium dissociation constants of 1.7 and 2.2 nM. In addition, the equilibrium dissociation constant of fluorescently labeled Ec4 to EpCAM-positive MCF-7 cells was measured on a flow cytometer, again from the ratio k_d/k_a . A slightly higher K_D of 5.8 nM was obtained from association ($k_a = 5.5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) and dissociation ($k_d = 3.2 \times 10^{-4} \text{ s}^{-1}$) rate constants (Supplementary Figure 1C and 1D).

Cytotoxicity of Ec4-ETA" against various tumor cell lines *in vitro*

The cytotoxic effect of Ec4-ETA" and the control fusion proteins off7-ETA" and E3_5-ETA" on various EpCAM-positive tumor cell lines and EpCAM-negative control cells was determined in colorimetric XTT cell viability assays upon a 72 h incubation. As shown in Fig. 2A, Ec4-ETA" was potently cytotoxic against all EpCAM-positive cell lines tested, MCF7, SW2, CAL27 and HT29. The IC_{50} values (concentration at which cell viability was reduced by 50%) ranged from less than 0.005 pM to 0.7 pM. In contrast, the IC_{50} value on EpCAM-negative RL cells was more than 100'000-fold higher ($IC_{50} > 10 \text{ nM}$). Similarly, the unspecific fusion proteins off7-ETA" and E3_5-ETA" showed cytotoxic effects only at much higher concentrations ($IC_{50} > 1 \text{ nM}$) than the EpCAM-specific ones when tested on EpCAM-positive HT29 cells (Figure 2B).

Furthermore, the cytotoxicity of Ec4-ETA" was markedly decreased when cells were preincubated with an excess of unfused DARPin Ec4 as competitor

(Figure 2C). This decrease was specific for EpCAM blocking, as preincubation with the non-specific DARPins off7 and E3_5 did not diminish Ec4-ETA" cytotoxicity. Moreover, the use of the unfused DARPins did not affect cell viability (data not shown). Taken together, these data show that the cytotoxicity is mediated by EpCAM-specific uptake and background cytotoxicity by unspecific uptake of the DARPIn-ETA" fusion proteins was in general very low.

Tumor localization of Ec4-ETA"

To demonstrate that Ec4-ETA" efficiently localizes to tumors upon systemic administration and that this effect is EpCAM-dependent, *in vivo* fluorescence imaging was performed in athymic mice bearing s.c. HT29 tumor xenografts. Ec4-ETA" and off7-ETA" control were N-terminally labeled with the fluorescent dye Cy5.5 (emission maximum 680 nm). After the coupling reaction, the labeled proteins were purified by anion exchange chromatography to eliminate unlabeled protein, free dye and other labeled protein species. Mice were injected i.v. with 30 µg Ec4-ETA"_Cy5.5 or off7-ETA"_Cy5.5, and images were taken after 6, 24, 48, 72 and 96 h using the NightOWL II LB891 imaging system. As shown in Fig. 3A, 6 h after injection both fusion proteins localized to the lower abdomen which could be identified as mainly kidney and partially liver accumulation. Ec4-ETA" efficiently localized to tumors 24 h after injection, and accumulation peaked between 48 h and 72 h before it declined to background values after 96 h. In contrast, only very low background fluorescence was detectable in the tumors upon injection of the non-targeted fusion protein off7-ETA".

Furthermore, we performed *ex vivo* analysis of biodistribution in isolated organs and tumors 48 h after injection of the fluorescent-labeled probe using a fiber optic device. As shown in Fig. 3B, similar to whole animal imaging, high fluorescence activity in the tumor was detected only in mice injected with Ec4-ETA" but not off7-

ETA". Probably as a consequence of metabolic degradation, both fusion proteins also showed localization in parts of the liver and at low levels in the intestine. These signals, however, were covered by overlaying tissue and thus undetectable by *in vivo* imaging.

Serum half-life of Ec4-ETA" in mice

To determine the serum half life ($t_{1/2}$) of Ec4-ETA", mice were injected with a single dose of 30 μ g and blood was collected at intervals between 3 and 60 min. The concentration of Ec4-ETA" in mouse serum was measured by sandwich ELISA. Each data point used to determine half-life represents the average of samples of 2 to 4 mice. Data were fit to a single exponential decay function with plateau (Supplementary Figure 2) and the serum half-life of Ec4-ETA" was determined as 11.2 min.

Anti-tumor effect of Ec4-ETA"

To investigate how the favorable tumor localization of Ec4-ETA" translates into therapeutic efficacy, its anti-tumor effect was evaluated in athymic mice bearing established s.c. tumor xenografts. As a first tumor model, we used the cell lines HT29. In one group, mice received 3 doses of 30 μ g Ec4-ETA" (Ec4-ETA" 30/3) on days 1, 3 and 5. In a second group, 6 doses of 20 μ g Ec4-ETA" (Ec4-ETA" 20/6) were administered on days 1, 3, 5, 9, 13 and 15. Control mice received PBS or 3 doses of 30 μ g off7-ETA" (off7-ETA" 30/3) on days 1, 3 and 5.

As shown in Figure 4A, tumors of control mice treated with PBS grew rapidly until the end of the observation period. In contrast, in all mice treated with Ec4-ETA" tumor growth was strongly inhibited. Tumors of mice treated with the Ec4-ETA" 30/3 schedule almost completely disappeared after the last injection, but started to regrow when treatment was discontinued. Nonetheless, in this group 2 of 11 mice (18 %)

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showed complete regression, defined as non-detectable tumor or no tumor regrowth for more than 45 d. The anti-tumor effect of the Ec4-ETA" 20/6 schedule was even more pronounced, resulting in 2 of 5 mice (40 %) with complete regressions. Lower doses of Ec4-ETA" (5 µg) did not result in significant tumor responses (data not shown). Treatment with the control fusion protein off7-ETA" 30/3 had no effect on tumor growth compared to mice treated with PBS. To better discriminate between the response rates in the various groups, Kaplan-Meier curves were plotted with an end point defined as a tumor size > 100 mm³. As shown in Figure 4B, all control mice treated with PBS or off7-ETA" developed tumors > 100 mm³ already 9 d after the start of treatment. At this time point, all mice in the Ec4-ETA"-treated group had tumors clearly below this size. On day 31, 18 % of mice treated with Ec4-ETA" 30/3 and 60 % of mice treated with Ec4-ETA" 20/6 still showed tumors < 100 mm³, indicating that treatment with the Ec4-ETA" 20/6 schedule was more effective in tumor control than with Ec4-ETA" 30/3. At the end of the experiment (day 31), the average size of tumors was significantly reduced from 1005 ± 275 mm³ (PBS group) to 188 ± 125 mm³ in the Ec4-ETA" 30/3 treated group ($P < 0.05$) and to 89 ± 87 mm³ in the Ec4-ETA" 20/6 treated group ($P < 0.05$). This reflects a reduction in tumor volume of 81 % and 91 %, respectively. The favorable effect of the Ec4-ETA" 20/6 schedule, which delivered a higher overall dose but without increased toxicity, is also reflected by the 40 % complete regressions (see above). The high standard deviations observed for Ec4-ETA" are due to the fact that some mice showed complete regressions. No difference in tumor size was found in mice treated with off7-ETA" 30/3 compared to PBS.

We confirmed the anti-tumor activity of Ec4-ETA" in a SW2 small cell lung carcinoma xenograft model, which shows a slower growth rate and is known to form a very solid tumor mass. Mice bearing s.c. SW2 tumors were treated with 6 doses of 20 µg Ec4-ETA or off7-ETA" on days 1, 3, 5, 8, 10, and 12. As shown in Figure 4C,

treatment with Ec4-ETA" decreased the tumor size compared with controls (PBS and off7-ETA). On day 31, mice treated with Ec4-ETA" had an average tumor size of $257 \pm 114 \text{ mm}^3$ whereas mice treated with off7-ETA" and PBS already reached tumor sizes of $856 \pm 198 \text{ mm}^3$ and $776 \pm 214 \text{ mm}^3$, respectively. There was thus a significant reduction in tumor volume of treated compared to PBS control mice ($P < 0.05$), although the effect was slightly less pronounced than on faster growing HT29 tumors.

Toxicity of Ec4-ETA"

To determine treatment-related unspecific toxicity upon Ec4-ETA" and off7-ETA" administration, mice were monitored for weight loss, dehydration and signs of distress (apathy, hyperalgesia and ungroomed appearance) throughout the course of the study. Representative for all therapy experiments Figure 5 shows for HT29 tumor-bearing mice that treatments were well-tolerated and after reversible marginal weight loss after the third injection of Ec4-ETA" 30/3 no further signs of toxicity were observed. Furthermore, to assess liver toxicity as a frequent dose-limiting side effect of ETA fusion toxin therapy in patients, blood from 4 PBS-treated mice and 4 mice receiving 3 doses of $30 \mu\text{g}$ Ec4-ETA" was collected and analyzed for activity of the liver transaminases aspartate aminotransferase (AST) and alanine aminotransferase (ALT). As shown in Table 1, liver toxicity could be excluded for this treatment schedule, as there was no significant elevation of ALT and AST activity in the plasma of Ec4-ETA"-treated (ALT: $54 \pm 22 \text{ U/L}$, and AST: $141 \pm 47 \text{ U/L}$) compared to PBS-treated mice (ALT: $57 \pm 51 \text{ U/L}$, and AST: $99 \pm 53 \text{ U/L}$).

Discussion

Chemotherapy still has remained the mainstay of cancer therapy. The great majority of approved treatments have no inherent specificity for tumor cells but attack all dividing cells. More recently many investigations have been carried out to couple a toxic principle to a recognition function. Immunotoxins based on *Pseudomonas aeruginosa* exotoxin A (ETA, also termed PE), particularly its truncated variant carrying a C-terminal KDEL peptide (ETA"), have been generated against various cell surface receptors and extensively tested in preclinical and early clinical studies (5,34). Most of them use a single-chain fragment (scFv) or a disulfide-stabilized dsFv fragment of antibodies as targeting moiety. We previously also reported on the potent anti-tumor effect of an EpCAM-specific scFv-ETA" immunotoxin (11), which is currently under phase II clinical investigation (9).

Commonly used antibody Fv-based formats, however, are difficult to produce in high amounts when compared to other proteins and are often aggregation-prone (24,26,35). ScFv and dsFv fragments used for tumor-targeted fusion proteins must either be expressed in the periplasm or refolded after expression in inclusion bodies. Here, we investigated another class of highly advanced binding molecules, Designed Ankyrin Repeat Proteins (DARPs), for targeted delivery of ETA" to tumor cells *in vitro* and *in vivo*. DARPs can be selected for high affinity, which is a major requirement for efficient tumor targeting (36), their inherent robustness allows easy conjugation with various types of effector molecules, and pharmacology and tumor targeting properties can be easily modulated, e.g. by site-specific PEGylation. Therefore, we are not limited to the simple fusions described here, as the molecules are robust enough to allow efficient production of more complicated constructs. As shown recently, the possibility to produce DARPs in functional form in multi-gram

amounts by fermentation of *E. coli* and their easy purification can significantly reduce the time from preclinical development to clinical investigation²

We are expressing the DARPIn-ETA" fusions in the cytoplasm of *E. coli* and find that these fusion proteins are produced in soluble form. Even though the ETA part contains two disulfide bonds, which appear to be beneficial in the internalization process (37), we find in the purified protein that they have been formed almost quantitatively, possibly by air oxidation. Thus, we can use the convenient production of DARPins in the *E. coli* cytoplasm also with the DARPIn-ETA" fusion proteins.

A major challenge for the application of immunotoxins is the choice of targets which provide sufficient tumor specificity and, at the same time, promote intracellular delivery of the payload. Several targets have been explored which serve this purpose, e.g. EGFR, LewisY, and various hematopoietic markers (2). Most tumor-targeting with fusion toxins has been studied in hematopoietic malignancies, but even there, only one (denileukin diftitox, Ontak[®]), a fusion of IL-2 with diphtheria toxin, finally received FDA approval (38).

EpCAM is overexpressed in many solid tumors and on the basolateral cell surfaces of some normal epithelia (15,18) where it is, however, poorly accessible to circulating anti-EpCAM antibodies (17). Since it also occurs on normal hepatic stem cells and hepatoblasts, EpCAM-targeted immunotherapy may in principle impair liver regeneration. However, liver toxicity has never been reported in clinical trials with anti-EpCAM antibodies, probably because the regenerative capacity of the adult liver is sufficiently maintained by mitotic division of mature hepatocytes (39). In contrast, the inherent liver toxicity of ETA is based on inflammatory responses caused by liver macrophages, independent on specific hepatocyte targeting (40).

² <http://clinicaltrials.gov/ct2/show/NCT01042678>,

<http://clinicaltrials.gov/ct2/show/NCT01086761>.

Recently, EpCAM was also identified as a marker of cancer-initiating cells in colon (21), breast (22), pancreatic (23) and hepatocellular carcinomas (24). Since cancer stem cells respond poorly to standard therapy and thus are largely responsible for treatment failure, their elimination must be a prime objective for all kinds of innovative cancer therapy. Thus, from a therapeutic point of view, EpCAM is particularly interesting as a docking site for targeting ligands delivering external effector molecules. In fact, EpCAM efficiently mediates internalization of bound ligands by receptor-mediated endocytosis and thus perfectly matches the need of anti-cancer agents acting on intracellular targets, such as protein toxins, chemotherapeutic agents, and antisense compounds (11-13).

Recently, we have described for the first time the production and biochemical characterization of EpCAM-specific DARPins, and a first generation binder was used to successfully deliver therapeutic siRNA into tumor cells in the form of mono- and multivalent binders fused to highly charged protamine (14). Subsequent affinity maturation efforts then resulted in DARPIn Ec4, which displayed affinity to EpCAM in the nanomolar range and excellent biophysical properties¹.

We measured efficient tumor localization of Ec4-ETA", but not of the control fusion off7-ETA", by *in vivo* fluorescence imaging upon systemic administration in a HT29 colon carcinoma xenograft model. These data showed that localization in the tumor was specific and dependent on EpCAM binding with peak tumor accumulation 48-72 h after injection.

Fluorescent imaging records the distribution of molecules at any particular time point, while the previously used residualizing label ^{99m}Tc(CO)₃ (36) gives an integral localization since the begin of injection, since any label will accumulate at the site of cellular internalization. This probably accounts for the difference in kidney accumulation, which is seen to decay rapidly when measured by fluorescent imaging but not by ^{99m}Tc(CO)₃ radioactivity. While immunotoxins (scFv fragments or DARPins

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fused to a protein toxin such as ETA) would be expected to not fall in an ideal MW range for maximum accumulation (36), we see very encouraging enrichments and therapeutic effects already with the constructs described here, and there may be even better effects with different molecular formats.

In the present study, Ec4 was fused with ETA" to assess for the first time the potential of a rationally engineered DARPIn for tumor targeting and therapy. The fusion protein could indeed be well produced in soluble form in *E. coli* at about 80-fold higher yield from shake flasks than our previously described scFv-ETA" fusion toxin (4D5MOCB-ETA) (11), could be easily purified and was stable and resistant to aggregation. This will facilitate subsequent scaling up of the production process required for clinical trials.

We found that the high-affinity binding of Ec4 to intact cells was fully preserved upon fusion with ETA" and that Ec4-ETA" showed extremely high and specific *in vitro* cytotoxicity against EpCAM-positive tumor cells of various histotypes with IC₅₀ values of 0.005 pM. This is remarkably low compared to other immunotoxins (41-43) and likely mirrors its high stability, affinity and efficient internalization by receptor-mediated endocytosis. These data, combined with efficient tumor targeting, indeed translated into potent anti-tumor effects at well-tolerated doses with some mice showing complete regression of their tumors. The higher overall dose administered as 6 x 20 µg could prove more effective than the lower dose schedule (3 x 30 µg). Probably due to the short half-life of the DARPIn-ETA" fusion toxin in the circulation which may limit tumor accumulation but also toxicity to normal tissues, the dose required to achieve tumor responses was higher than for a previously described scFv-ETA" immunotoxin (11). The repeatable response measured after repeated injections further suggests that tumors retained a stable EpCAM expression profile and that antigen loss did not occur during treatment.

A phase II study with this previously reported EpCAM-specific immunotoxin (11) consisting of ETA" fused to a scFv antibody is ongoing and will be completed soon (9). Based on the findings of the present study, it is tempting to speculate that DARPins like Ec4 can replace the antibody fragment as cell binding ligands also in forthcoming generations of tumor-targeted fusion toxins as well as other drug delivery systems.

In summary, we describe for the first time the generation and preclinical evaluation of an EpCAM-specific fusion toxin consisting of a high-affinity DARPIn (Ec4) and ETA" as a catalytic biotoxin. We provide evidence for its potent activity against various tumor cell types *in vitro*, and its favorable tumor localization and anti-tumor activity *in vivo*. The advantages of DARPins enabling high yield expression, resistance against aggregation, and stability also in the form of fusion toxins in conjunction with a tumor-associated target like EpCAM, opens new avenues for the generation of rationally designed protein therapeutics with outstanding efficacy.

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Acknowledgements

This work was supported by the Swiss National Science Foundation (grant 310030_119859) and the Krebsliga of the Kanton Zürich. We thank Dr. Beat Kunz and Gabriela Nagy-Davidescu for their support with animal experiments, Dr. Ykelien Boersma for helpful discussions and Annemarie Honegger for modeling the structure of Ec4-ETA".

Figure Legends

Figure 1. Biochemical characterization of the DARPIn-ETA⁺ fusion toxins. **A** For SDS-PAGE analysis, fractions were loaded onto a 10% polyacrylamide gel and proteins were detected by staining with Coomassie brilliant blue: lane M: molecular weight marker (kDa), lane 1: uninduced bacterial cell lysate, lane 2: cell lysate 4 h after induction, lane 3: purified Ec4-ETA⁺, lane 4: purified off7-ETA⁺, and lane 5: purified E3_5-ETA⁺. **B** Size exclusion chromatography of DARPIn-ETA⁺ fusion toxins analyzed on a Superdex 200 10/300 GL column. **C** Model of the DARPIn-toxin construct was built based on the X-ray structures of a consensus DARPIn (PDB entry 2QYJ) and *Pseudomonas aeruginosa* exotoxin A (ETA) (1IKQ) using InsightII (Accelrys) and the ROSETTA suite programs. The DARPIn targeting moiety is shown in cyan, ETA domain Ib in purple, domain II in orange and domain III in red. The flexible N-terminal RGS-His₆-tag, the C-terminal His₆ tag and KDEL ER-retention signal (blue) as well as the linker connecting the DARPIn to the toxin (green) were modeled in to visualize their sizes relative to those of the protein domains. The figure was generated using the program PyMol (DeLano Scientific LLC, Palo Alto, CA, USA).

Figure 2. *In vitro* cytotoxicity of Ec4-ETA⁺ and the control fusion toxins off7-ETA⁺ and E3_5-ETA⁺ tested on various tumor cell lines. **A** The EpCAM-positive cell lines MCF7, SW2, CAL27, HT29, and the EpCAM-negative cell line RL were incubated for 72 h with different concentrations of Ec4-ETA⁺ before cell viability was determined in colorimetric XTT assays. **B.** HT29 cells were incubated with the unspecific fusion proteins E3_5-ETA⁺ or off7-ETA⁺ for 72 h in XTT assays, and viability was compared to cells treated with Ec4-ETA⁺. **C** In competition assays, EpCAM-positive HT29 cells were incubated with DARPIn Ec4, E3_5 and off7 (500 nM) 10 min before Ec4-ETA⁺

was added and cell viability determined as described above. All determinations were done in triplicates, data represent the mean \pm SD

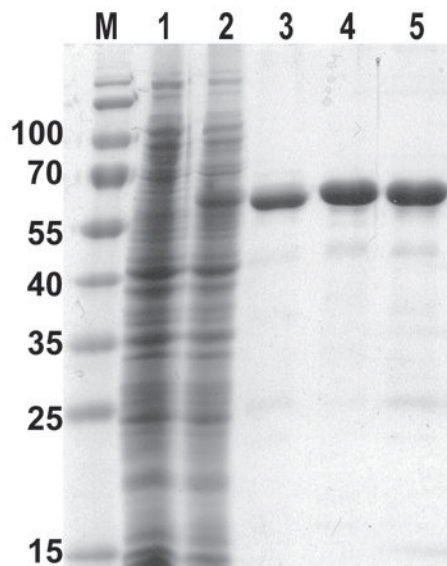
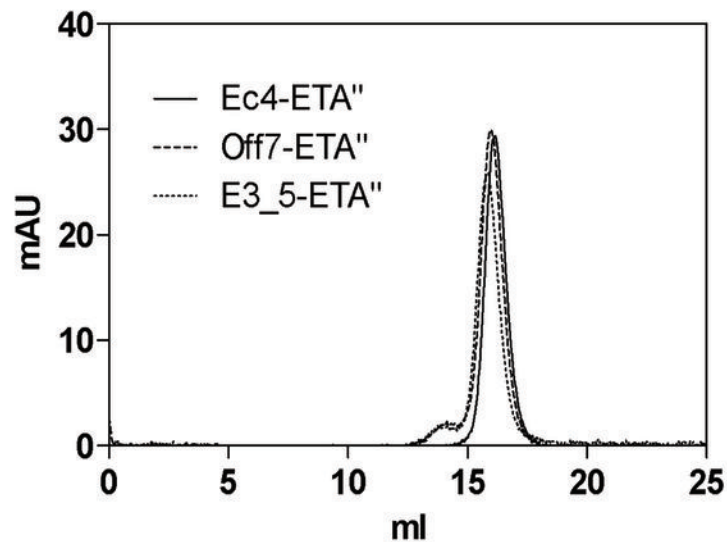
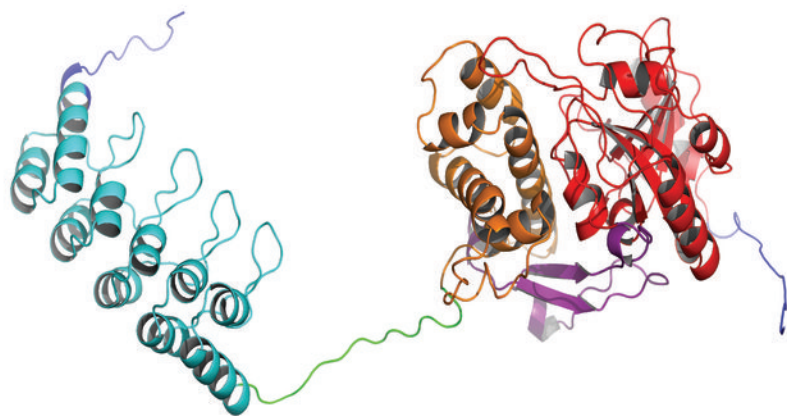
Figure 3. Tumor localization and organ distribution of Cy5.5-labeled Ec4-ETA" and the off7-ETA" control fusion toxin detected by *in vivo* fluorescence imaging. Mice bearing s.c. HT29 tumor xenografts were intravenously (i.v) injected with 30 μ g of Ec4-ETA" or off7-ETA" conjugated with the fluorescent dye Cy5 (n=3 for each group). **A** *In vivo* images were acquired 6, 24, 48, 72, and 96 h after injection using the NightOWL II LB891 imaging system with an exposure time of 60 s. For colocalization of the fluorescent image on the animal body, gray scale and pseudocolor images were merged. ND: not determined. **B** *Ex vivo* analysis of fluorescence intensities from isolated tumors and organs 48 h after injection of Cy5.5-labeled Ec4-ETA" or off7-ETA" using a fiber optic device. Organs of one representative mouse of each group is shown. Data are plotted as the average of two mice; bars, SD

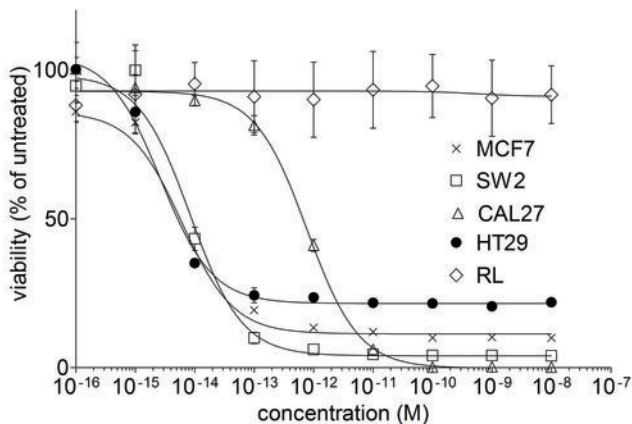
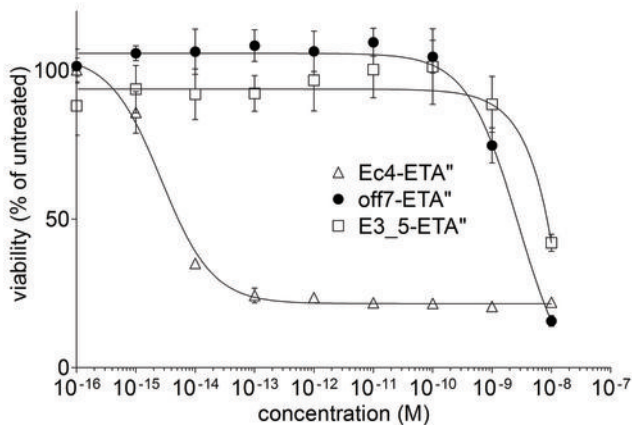
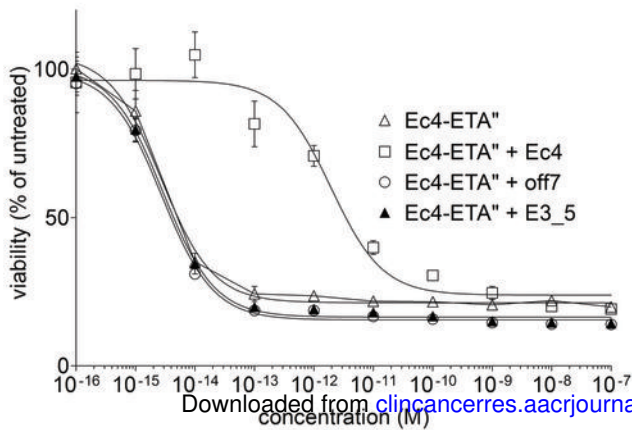
Figure 4. Anti-tumor effect of Ec4-ETA" in athymic mice. **A.** Mice bearing s.c. growing HT29 tumor xenografts of 50 to 100 mm³ in size received tail-vein injections of either 3 x 30 μ g Ec4-ETA", 3 x 30 μ g off7-ETA" control, 6 x 20 μ g Ec4-ETA" or PBS as vehicle control. Tumor growth was monitored by caliper measurement during the course of 31 days. Data represent the mean tumor volume \pm SD of 5 to 11 mice per group. **B** Kaplan-Meier survival curves with an endpoint defined as tumor volume of 100 mm³. The curves show the percentage of treated mice in each group in which tumors did not exceeded 100 mm³ in size at the various time points after the start of treatment. **C** Mice bearing s.c. growing SW2 tumor xenografts of 40 to 80 mm³ in size received tail-vein injections of either 6 x 20 μ g Ec4-ETA", 6 x 20 μ g off7-ETA" control, or PBS as vehicle control. Tumor growth was monitored by caliper measurement

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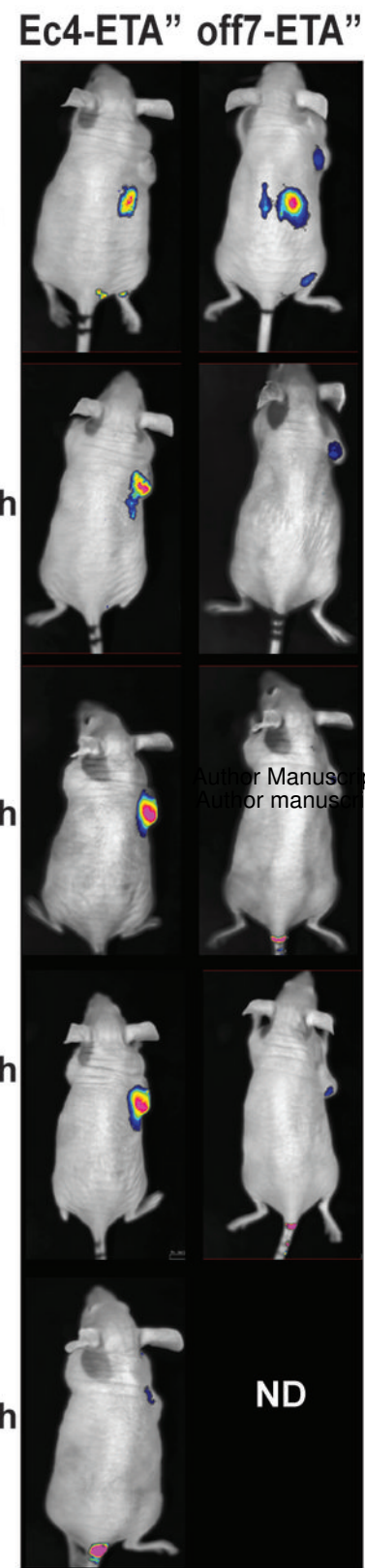
during the course of 31 days. Data represent the mean tumor volume \pm SD of 5 to 6 mice per group. **D** Pictures of representative tumors isolated from mice of the different treatment groups.

Figure 5. Average weight of mice upon treatment with different dose schedules of Ec4-ETA", or off-7-ETA" or with PBS control. Animals were weighed three times per week during the whole experiment to monitor treatment-related toxicity.

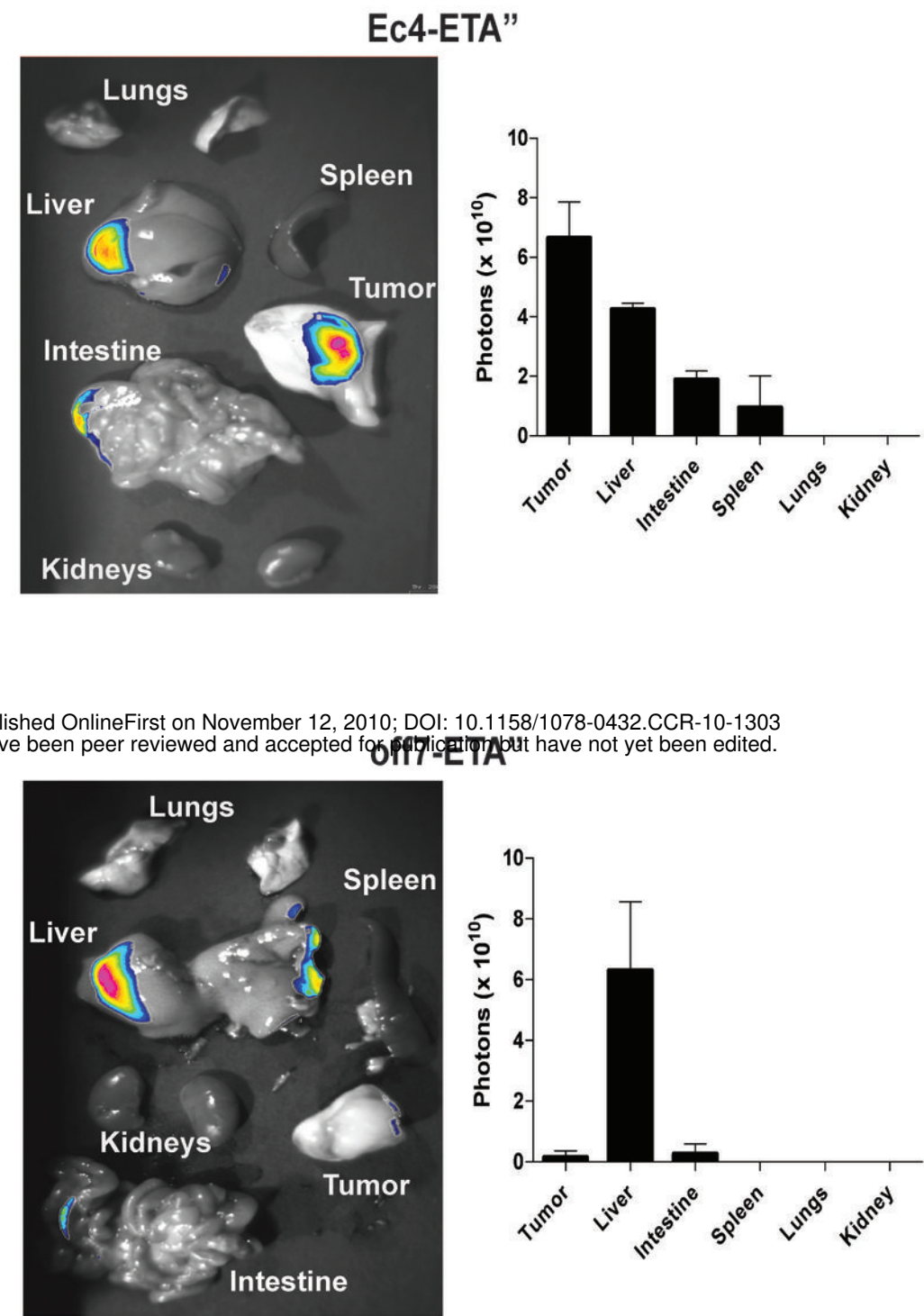
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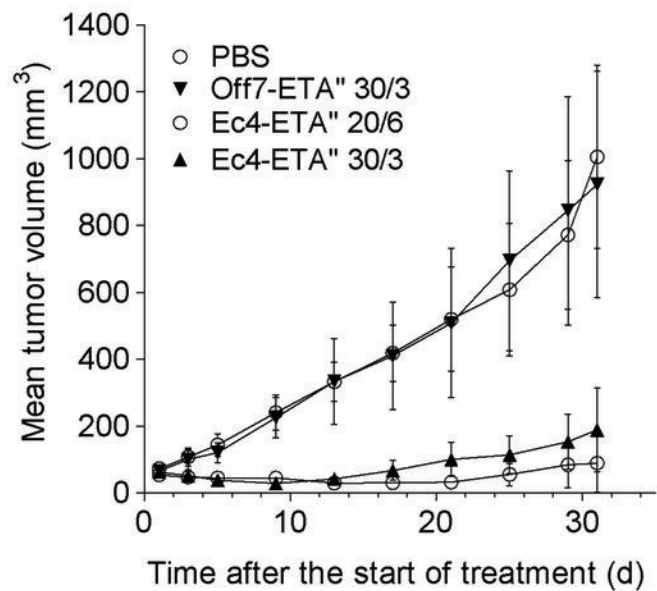
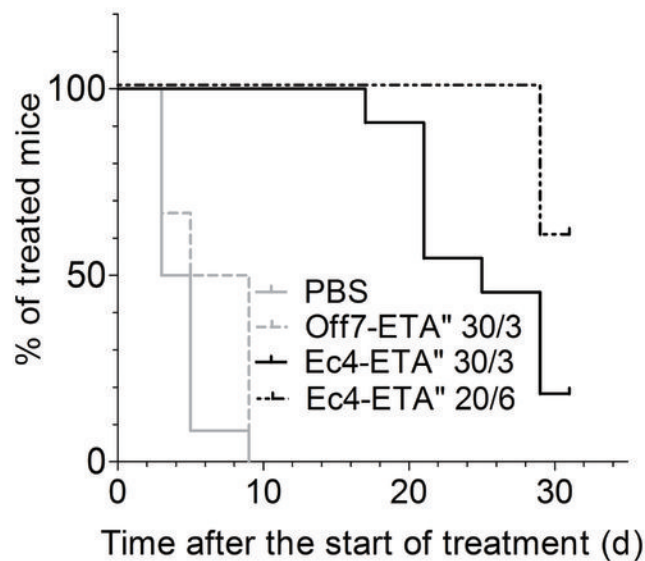
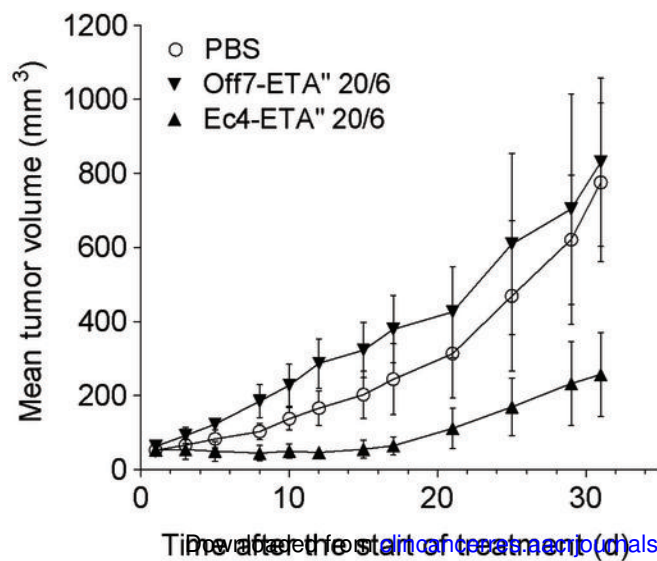
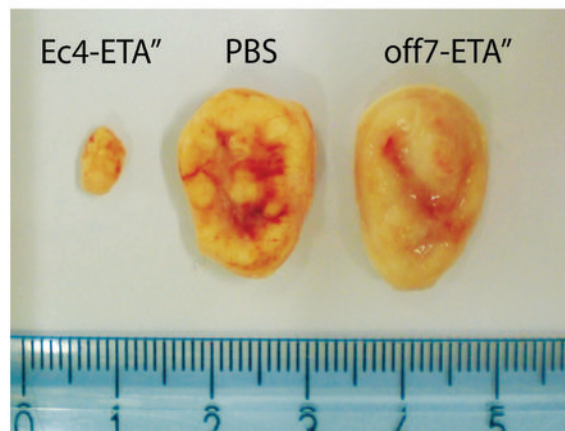
A



B



Author Manuscript Published OnlineFirst on November 12, 2010; DOI: 10.1158/1078-0432.CCR-10-1303
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

A**B****C****D**

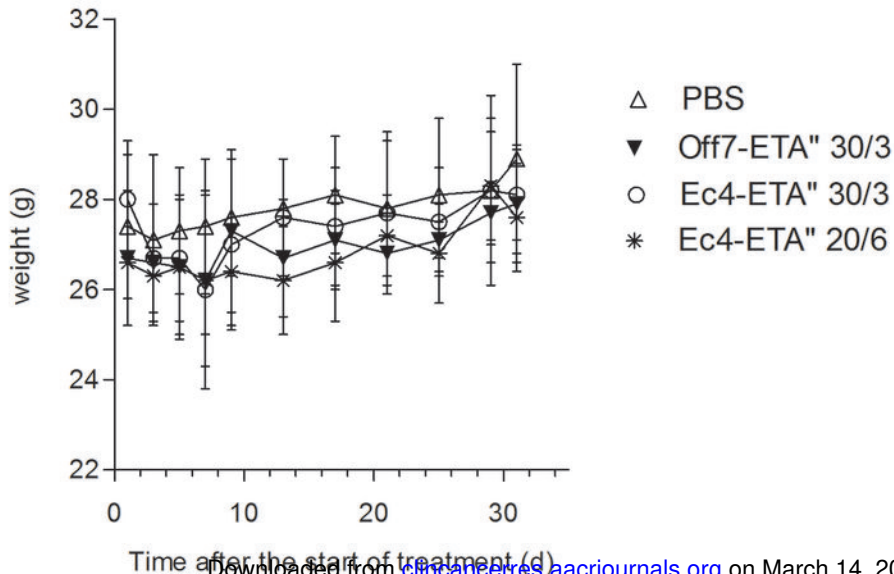


Table 1. Liver transaminase activity in the plasma from mice treated with Ec4-ETA”

	Treatment *							
	Ec4-ETA”				PBS			
	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 1	Mouse 2	Mouse 3	Mouse 4
ALT U/L	34	84	57	41	134	40	26	29
AST U/L	141	200	138	85	177	62	86	70

*Mice received 3 x 30 µg of Ec4-ETA” or PBS i.v. Activity of the transaminases was measured 24 h after the final injection.

ALT, alanine aminotransferase; AST, aspartate aminotransferase.